AD_____

Award Number: DAMD17-02-1-0278

TITLE: The Role of Estrogen Receptor a K303R Mutation in Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Yukun Cui, Ph.D.
Suzanne Fuqua, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (6 May 2003 - 5 May 2004)

4. TITLE AND SUBTITLE
The Role of Estrogen Receptor a K303R Mutation in Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-02-1-0278

6. AUTHOR(S)
Yukun Cui, Ph.D.
Suzanne Fuqua, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: ycui@breastcenter.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Last year we reported that MTA1-L1 is an ER-corepressor but unable to repress the activity of the naturally occurred K303R ER mutant. We report here that MTA1-L1 could deacetylate the p300 acetylated ER, probably via its associated HDAC activity. Also we found that a HDAC inhibitor reverses MTA1-L1 inhibited ER activity, suggesting that MTA1-L1 inhibit ER activity through HDACs. Later we determined that two other corepressors, NcoR and BRCA1, which also associated with HDAC, can not repress the K303R activity, suggesting that the hypoacetylated state of K303R might account for its ligand hypersensitivity. However, using different mutations in the K303 sites, we demonstrated that hypoacetylation is not sufficient for K303R ER hypersensitivity, suggesting that other signals such as kinases could play a role. In deed, we found that K303R mutant makes ER Ser305 a more efficient target for both Protein Kinase A(PKA) as well as p21-activated kinase 1(PAK1) through both in vitro and in vivo kinases assays. Also, phosphorylation of Ser305 can inhibit K303 acetylation by p300. In addition, mimic Ser305 phosphorylation by Ser305Asp mutation confers ER hypersensitive to estrogen. In summary, our data demonstrated that K303R alters ER regulation by multiple signals.

14. SUBJECT TERMS
ERα, PKA, p300

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTROUCTION

It is known that estrogens play a key role in regulating the proliferation of normal breast epithelium, as well as breast cancer, through binding with their cognate receptors (ERs), which belong to a superfamily of nuclear steroid receptors and function as transcriptional factors to mediate the mitogenic effects of estrogens. It has been reported (1,2) that less than 10% normal breast epithelium express ERα, but that about 60% of UDH and 60-70% of breast tumors express ERα, so it is felt that the expression and/or activation of ER influences both breast carcinogenesis and progression. There are two current models for ER activation. One is through its cognate ligand binding; the other involves alternative pathways such as phosphorylation, acetylation, or mutation. Recently our group (3) has discovered that a mutated estrogen receptor called K303R, is correlated with both breast cancerignesis and development, and also this mutant is a hypoacetylated ER form, so that we proposed to study the effect of an HDAC-associated protein, MTA1-L1 mediated deacetylation of wild-type ER function, as well as to study the effects of expressing the K303R mutant on breast cancer cell metastatic behavior in the following Specific **Aims**:

Aim 1. To elucidate the relationship between MTA-L1 expression, and ERα protein acetylation as well as ERα activity: We have already shown an *in vitro* interaction between wild-type ERα and MTA-L1 using GST-pull down experiments. To detect the potential significance of this interaction, we will examine for *in vivo* interactions as well as the effects of ERα agonists and antagonists on this interaction using further GST-pull down and co-immunoprecipitation assays. The effects of MTA-L1 expression on ERα transcriptional activity will also be determined using transient transactivation assays and estrogen-responsive reporter assays. The effects of MTA-L1 expression on ERα acetylation status will be determined using *in vitro* deacetylation assays.

Aim 2. To study the metastatic process influenced or driven by the K303R ERα mutation, and to develop a somatic knock-out model of the ERα gene in the T47D cell line to study the *in vivo* effects of the K303R mutation. A somatic knock-out of the human ERa gene will be performed using standard approaches targeted to the amino region or the DNA binding domain. The functional significance of the ERα mutation will first be determined using *in vitro* motility and invasion assays of the knock-out cell line transfected with an expression vector for the wild-type, the K303R mutant, or co-expressed MTA-L1. The *in vivo* metastatic potential and behavior of the transfected lines will then be examined using both ectopic growth and tail vein injection into athymic nude mice. Metastatic lesions, if present, will be examined for WT ER, K303R ER, nd/or MTA-L1 expression using microdissection, genomic sequence, and SNP analyses. The sensitivity of the metastatic lesions to estrogens antiestrogens will be examined to determine their hormonal response to treatment with these agents.

BODY

Specific Aim 1

MTA1-L1 deacetylates acetylated ERα– Last year, we reported that we had completed the major part of Aim1. For the past year, we generated T47D cells overexpressing MTA1-L1 (Fig

1, Panel A), and found that both ER and HDAC1 are present in MTA1-L1 complexes (Fig 1, Panel B), eventually we determined that MTA1-L1 could deacetylate ERα protein (Fig 1, Panel C).

A HDAC inhibitor could reverse MTA1-L1-repressed ERα activity- we performed transient Tran activation experiments to determine whether inhibition of MTA1-L1 associated HDAC inhibitor could release MTA1-L1-inhibited ER activity and found, sodium butyrate, a wildly used HVACs inhibitor could reverse MTA1-L1 repressed wild-type ER activity (Figure 2).

K303R mutant is also refract to the inhibition of Nor and BRCA1 – Our data suggest that it might be MTA1-L1-associated HVACs activity which mediate MTA1-L1 inhibition of wild-type ER. Since K303R ER is the non- or hypo acetylated ERα mutant which might be refractory to those HVACs mediated tranrepression. So we hypothesized that K303R mutation could also be refractory to other HDAC-associated corepressors. To address this question we performed transient transactivation assay to determine the effects of NcoR and BRCA1 on both wild-type and the K303R ER mutant, and we found that K303R ER is also refractory the inhibition of both NcoR (Fig 3, Panel A) and BRCA1(Fig 3, Panel B).

Hypoacetylation of K303 ERα does not elucidate the mechanism for the hypersensitive phenotype-

So far, our results suggest that the hypoacetylated state of the K303R mutant makes this mutant refractory to the inhibition of these corepressors. To test this hypothesis we generated the two other possible nucleotide substitutions (A to T or C), and tested their transcriptional activity in transient transactivation ERE-luciferase (Fig. 4, Panel A). Our result suggests that only the specific K303R substitution results in a receptor that is hypersensitive to hormone. Since K303 being the major acetylation site in ERα. *In vitro* acetylation assays demonstrated that all of the K303 substitutions were hypoacetylated (Fig.4, Panel B upper). We determined that equal amounts of proteins were assayed in the acetylation assays by Commassie Blue Staining (Fig. 4B, lower).

ERα protein acetylation and phosphorylation are inversely correlated – We next hypothesized that not only acetylation, but also kinases signals may play a role conferring the K303R mutant ligand hypersensitivity. Therefore, we incubated MCF-7 cells in either medium stripped and devoid of growth factors and estrogen, or in medium containing full serum for 48 hours prior to preparation of cell lysates. The cell extracts were then divided, and used for either a total *in vitro* kinase phosphorylation assay with GST-WT ERα as the substrate (Fig. 5A), or for an immunoprecipitation (IP) and immunoblot (IB) analysis (Fig. 5B). As expected, total ERα phosphorylation was increased with the addition of serum to the media (compare the – and + lanes, Fig. 6A). These results suggested that the processes of ERα phosphorylation and acetylation might be coupled in some way. We next examined whether mutations in the acetylation motif (K303R, K303M, or K303T) would affect the ability of ERα to serve as a substrate for *in vitro* kinase activity. We found that the specific K303R ERα mutation, but not the other two mutations K303M or T, was an efficient phosphorylation substrate, and furthermore that the K303R mutation compared to WT ERα might be a more efficient substrate for total kinase activity contained in MCF-7 cells (Fig. 5C). To address the location of the potential phosphorylation site within the ERα GST fragments used as substrates, we next

mutated a conserved serine at residue 294 to alanine to inactivate this site (S294A), and mutated the serine at residue 305 to aspartic acid to mimic the acidic charge of phosphorylation (S305D); these GST fragments were then used in *in vitro* kinase assays (Fig. 5D). We found that the S294A mutation had little effect on ERα phosphorylation, but that the S305D mutation greatly reduced the phosphorylation status of the ERα fragment. This result led us to next question which specific cellular kinase might be responsible for phosphorylation of ERα S305.

K303R ERα S305 is an *in vivo* and *in vitro* PKA site- The potential PKA site in WT ERα S305 (SKKNSL) has previously been noted(3), the K303R ERα mutation retains this potential PKA consensus site, therefore we first tested whether it could be directly phosphorylated by recombinant PKA in an *in vitro* kinase assay. Utilizing equal amounts of the various ER constructs as substrate (Fig. 6B, lower panel), we found that the K303R ERα mutant was the most efficiently phosphorylated, compared to WT or the K303 M and T mutants (upper panel). To confirm the S305 site as an authentic *in vivo* phosphorylation site, we transfected U2OS cells with expression vectors for the WT and K303R, as well as K303R,S305A ERs and metabolically labeled the cells with p32-orthophosphate and treated the cells with PKA activator 8-Br-cAMP. We found that K303R mutant generate Ser305 a more efficient PKA site *in vivo* (Fig. 6C). .

Modulation of PKA intracellular signaling influences the hormone sensitivity of ER– We next examine the effect of PKA on the activity of K303R mutant. A transient transactivation assay in Hela cells demonstrated that the WT receptor exhibited a similar magnitude of response to estrogen, irregardless of the addition of the PKA activator 8-Br-cAMP; in contrast, the transcriptional activity of the mutant receptor was enhanced at all concentrations of hormone tested (panel 7A). Treatment with the H89 PKA-selective inhibitor decreased estrogen-induced activity of both the mutant and WT receptors (panel 7B).

Phosphorylation of S305 mimics the estrogen hypersensitivity and hypoacetylation phenotype of the K303R ERα mutant. We compared the estrogen dose response of the phosphorylation mimic S305D in transient transactivation assays in a number of different cell lines and found that in all these cells the S305D ERα mutant exhibited an increase in estrogen sensitivity, suggesting that phosphorylation at this site is one determinant of hypersensitivity. When S305 was mutated to the non-phosphorylatable residue alanine (S305A), it exhibited a similar dose response as WT receptor, however, when we examined the activity of the double K303R/S305A mutant, we found that mutation of S305 abrogated the hypersensitivity of the K303R ERα mutant in breast cancer cells (Fig. 8A, panel D). In vitro acetylation assay also demonstrated that the phosphorylation mimic S305D ERα mutation exhibited significantly reduced acetylation *in vitro*. We conclude that a coupled cascade of post-translational ERα modifications might exist which are key determinants of estrogen sensitivity and transcriptional response.

Specific Aim 2

Overexpression of MTA1-L1 inhibites estrogen-stimulated, breast cancer cells proliferation- Since MTA1-L1 is an ER corepressor; we determined to study whether MTA1-L1 could inhibit breast cancer cells estrogen-induced proliferation. Using MTA1-L1 overexpressing T47D cells and vector control cells, we performed an typical cell growth assay (3), and demonstrate that MTA1-L1 inhibit estrogen-induced proliferation (Fig. 9)

Overexpression of MTA1-L1 enhanced breast cancer cells anchorage-independent growth in an estrogen-independent manner – Since MTA1-L1 is a metastasis-associated protein, and breast cancer cells anchorage-independent proliferation is a key step for cell metastasis (5), we determined to study whether MTA1-L1 could enhance breast cancer cells anchorage-dependent proliferation. Soft-agar was used as the growth media to test the anchorage-independent proliferation of MTA1-L1 overexpressing T47D cells. As we predicted, MTA1-L1 does enhances T47D cell growth in soft agar but in an estrogen-independent manner (Fig.10).

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that MTA1-L1-complexes can deacetylate acetylated wild-type ERa
2. Demonstrated that inhibition of HDAC activity could reverse MTA1-L1 inhibition of ER activity
3. Demonstrated that both NcoR and BRCA1 are not sufficient to repress K303R ER activity.
4. Demonstrated that K303R mutant generates a novel PKA site
5. Demonstrated that MTA1-L1 inhibit estrogen-induced, ER positive breast cancer proliferation
6. Demonstrated that MTA1-L1 enhance breast cancer cells anchorage-independent growth in an estrogen-independent manner.

REPORTABLE OUTCOMES

1. (1) meeting abstracts included in the Appendix.
2. Figues and relevant figure legends, and development of plasmid constructs were included in Appendix

CONCLUSIONS

We have successfully completed the proposed **Specific Aim1**. Results on this project have generated (2) manuscripts ready for submission and (1) meeting abstracts. We want to state that we have dramatically extended the studies proposed in **Specific Aim 1,** and generated more additional exciting results with potential translational significance. One meeting abstract based on this extended study was selected as a slide presentation in 2003 AACR annual meeting in Washington DC, 2003. Therefore, we have switched our major efforts to study multiple kinases signals such as PKA, AKT, and PAK1 on this very important, naturally occurred ER mutant. In addition, we also successfully finished some *in vitro* studies proposed in **Specific Aim 2**. The proposed studies will continue to be studied in the laboratory of the mentor, Suzanne A.W. Fuqua. Undoubtedly, the funds for this fellowship have been well spent with much success. It is also conceivable that this line of study will lead to direct clinical benefit.

REFERENCES

1. Peterson OW, Hoyer PE, and van Deurs B. *Cancer Res.* 47: 5748-5751, 1987
2. Jacquemier JD, Hassoun J, Torrente M, and Martin PM. *Br can Res Treat* 15:109-117, 1990
3. Fuqua SAW et al. *Cancer Res* 60:4026-4029,2000
4. Fiol, C. J., Williams, J. S., Chou, C. H., Wang, Q. M., Roach, P. J., and Andrisani, O. M. J Biol Chem, *269:* 32187-32193, 1994.
5. Mazumdar A, Wang RA, Mishra SK, Adam L, Bagheri-Yarmand R, Mandal M, Vadlamudi RK, Kumar R. Nat Cell Biol.3 (1):30-7,2001

APPENDICES

Reprints 1: Yukun Cui, Mao Zhang, Mai van, Torsten Hopp, Sara Sukumar, and Suzanne AW Fuwua. The K303R ER alpha breast cancer-specific mutation resides at a site of coupling ER acetylation and phosphorylation. *Proc. Amer Asso Cancer Res* 44:25, 2003

Construction of Plasmids- K303T, K303M, S305A, S305D, and S294A ERα constructs were generated using QuickChange Site-Directed Mutagenesis (Strategene, La Jolla, CA) to generate the indicated point mutations in pcDNA3.1-HA-WT ERα. The primer sequences for construction of the point mutations are as follows, with the mutated nucleotides underlined:
K303M, GATCAAACGCTCTAAGATGAACAGCCTGGCCTTG;
K303T, GATCAAACGCTCTAAGACGAACAGCCTGGCCTTG;
S305A, GCTCTAAGAA(G)GAACGACCTGGCCTTGTCCTGACG;
S305D, GCTCTAAGAA(G)GAACGCCCTGGCCTTGTC CTGACG;
S294A,GCCAACCTTTGGCCGACCCCGCTCATGATCAAACG;
K303R/S305A, GATCAAACGCTCTAAGAGGAACGACCTGGCCTTG;
K303R/S305D, GATCAAACGCTCTAAGAGGAACGCCCTGGCCTTG .
The entire nucleotide sequence of the construct was then verified by standard DNA sequencing procedures. The bacterial expression vectors for GST fusion proteins of the ERα hinge region (residues 253-310) were constructed by cloning PCR-amplified fragments derived from their respective mammalian vectors. To generate CMV-promoter drive GST-fused ER hinge vectors, GST-cDNA was amplified from GST 4T-1 and inserted into pcDNA3.1 vector through Hind III and Bam H1 sites, and the PCR-amplified fragments of different derived from their respective mammalian vectors were inserted into this vector through Bam H1 and EcoR I sites.

Figure Legends

Fig. 1. (A). Construction of T47D cells overexpressing MTA1-L1. Flag-tagged MTA1-L1 or control vector were transfected into T47D cells, G418 – resistant colonies were picked up to grow separately, and cell lysates from vector control clone and MTA1-L1 clone were resolved by electrophoresis. Anti-PID polyclonal antibody was used to detect the expression of Flag-MTA1-L1. Overexpressed MTA1-L1 as well as the endogenous MTA1-L1 were indicated with arrows. (B). Both HDAC1 and ERα are contained in MTA1-L1 complexes. Cell lysates from T47D cells overexpressing MTA1-L1 were subjected to immunoprecipitation with either protein G sepharose or anti-M2 sepharose. The immunoprecipitates were eluted with 3x Flag peptides and resolved by electrophoresis. Anti-HDAC1 antibody was used to detect the presence of

HDAC1. Same membrane was then stripped and the presence of ERα was detected with anti-ERα antibody. (C). MTA1-L1 complexes deacetylates the *in vitro* acetylated ERα. 25 μg purified

GST-wtERα (Hinge) or K303R ERα (Hinge) were acetylated by 0. 4 μg purified p300HAT in the precence of 50 nCi ^{14} C- Acetyl-CoA. The acetylated proteins were reimmobilized onto glutathion beads and equilibrated to the deacetylation buffer condition. Then the beads were tested for deacetylation by incubation with 3X Flag peptides elutes. The reactions were resolved by electrophoresis and analyzed by both commassie blue staining and autoradiography.

Fig.2. Blockade of HDACs activity restore MTA1-L1 suppressed ERα transcriptional activity. Cotransfection of 1 μg ERE-tk-Luc, 100 ng expression vector of either wtERα or K303R ERα with 0.2 μg expression vector of either MTA1-L1 or empty vector into Hela cells and treated with 10^{-9} M E2 or 10^{-9} M E2 plus 1 mM sodium butyrate (NaB) for 18-24 hours. Luciferase activities were normalized to the cotransfected internal control of pCMV-β-gal. The data shown as the fold induction to E2 treatment and represents the mean ± SEM for 9 separate transfections

Fig.3. K303R mutant is also refractory to the inhibition of NcoR and BRCA1. (A). Cotransfection of 1 μg ERE-tk-Luc, 100 ng expression vector of either wtERα or K303R ERα with 0.25 μg expression vector of either NcoR or empty vector into Hela cells and treated with 10^{-9} M E2 for 18-24 hours. (B) Cotransfection of 1 μg ERE-tk-Luc, 25 ng expression vector of either wtERα or K303R ERα with 25 ng vector of either BRCA1 or empty vector into Hela cells and treated with 10^{-9} M E2 for 18-24 hours. Luciferase activities were normalized to the cotransfected internal control of pCMV-β-gal. The data shown as the fold induction to E2 treatment and represents the mean ± SEM for 9 separate transfections.

Fig.4. A) Cotransfected with 1 μg ERE_2-tk-luciferase reporter plasmid, plus 1 ng of the respective ERα expression plasmid, and 100 ng of CMV-β-galactosidase expression plasmid as an internal control. Twenty-four hours after transfection, wells were then treated with increasing amounts of E_2 (10^{-12} to 10^{-9} M), as described in the Figure Legends. Cells were harvested after 18-24 hours of treatment with hormone, and ER luciferase activity was normalized by dividing by the β-galactosidase acitivity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average +/- SEM and are representative of at least three independent experiments. B) Same amount of GST-wt ER Hinge or GST-ER hinge harboring different K303 mutations were acetylated by GST-p300 as we described in Fig. 1C.

Fig.5. MCF-7 cells were grown in either medium stripped and devoid of growth factors and estrogen, or in medium containing full serum for 48 hours prior to preparation of cell lysates. The cell extracts were then divided, and used for either a total *in vitro* kinase phosphorylation assay with GST-WT ERα as the substrate (Fig. 5A), or for an anti-acetylated lysine antibody immunoprecipitation (IP) coupled immunoblot (IB) with antibodies against either ERα or AIB1 using analysis (Fig. 5B). To detect the total cellular activity of ERα as a specific kinase substrate, 20 μg of the respective GST-ERα hinge fragment was immobilized onto GSH-agarose beads, and after two initial washes in 500 μl kinase buffer (25 mM Tris HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$), the beads were incubated with 200 μg of MCF-7 cell lysate as described above, for 2 hours at 4° C. The beads were then washed three times with lysis buffer, two times in kinase buffer, and then the beads

were incubated with 10 nM ATP in the presence of 0.25 μCi gamma-^{32}P-ATP in 30 μl kinase buffer for 20 minutes at 30 °C. The reactions were stopped by the addition of SDS loading buffer, and boiled for 2 minutes. After a 2 minute centrifugation, the reactions were resolved on 4-12% SDS-PAGE gel, and the gels were visualized by autoradiography. C). Same amounts of GST-wtER or GST-ER harboring different K303 mutations were used for total kinase assay as described in Fig. 5A; D) Using same amount of GST-ER, GST-S294A ER and GST-S305D ER as substrate.

Fig.6. A), In vitro kinase assay were performed with same amount of GST-wtER or GST-ER harboring different K303 mutations as substrates, purified, recombinant PKA catalytic subunit as the kinase, kinase reaction were conducted as described in Fig. 5A; B), Firstly we transiently transfected CMV-GST-wt ER hinge, CMV-GST-K303R ER hinge, and CMV-GST-K303R/S305A Hinge into U2OS cells, 24 hours after transfection, cells were incubated in serum-free, phosphate-free DMEM media for two hours, and then incubated in phosphate-free DMEM supplemented with 5% dialysed fetal bovine serum at 37° C for another 4 hours, then 9.0 mCi 32p-orthophosphate were added with or without 10 μM 8-Br-cAMP were added, the cells were incubated for additional 4 hours. Then the cells were washed twice with ice-cold PBS and 0.5 ml hypertonic lysis buffer (400 mM NaCl, 20 mM Tris pH7.5, 50 mM NaF, 200 μM Sodium orthovanadate, 20 % Glycerol, and 1:100 diluted proteinase inhibitor cocktail) were put onto the plate, after 3 cycles freeze (on dry ice)-defreeze (on ice) . The cells were harvested and cleared with ultracentrifugation at 105,000 x g for 20 minutes. Then 1% Triton X100 and 1% Tween 20 were put into the cell lysates. The GST-ERs were pull down by incubation with 20 μl 50% GSH-agarose slurry at 4° C for 30 minutes. The beads were then washed 5 times with hypertonic lysis buffer plus 1% Triton X-100 and 1% Tween 20, and GST-ERs were eluted by boiling the beads in SDS loading buffer and resolved on 4-12% SDS-PAGE gel and transferred onto nitrocellular membrane. The 32p-labelled proteins were visualized by autoradiography(upper). GST-ERs were detected by western blotting with an anti-GST antibody (Lower).

Fig.7. Cotransfected with 1 μg E1b-ERE-TATA-luciferase reporter plasmid, plus 1 ng of the respective ERα expression plasmid, and 100 ng of CMV-β-galactosidase expression plasmid as an internal control. Twenty-four hours after transfection, wells were then treated with increasing amounts of E_2 (10^{-12} to 10^{-9} M), with 8-Br-cAMP(A) or H89. Cells were harvested after 18-24 hours of treatment with hormone, and ER luciferase activity was normalized by dividing by the β-galactosidase acitivity to give relative luciferase units. Experiments were performed in triplicate; the data showed is the representative of at least three independent experiments.

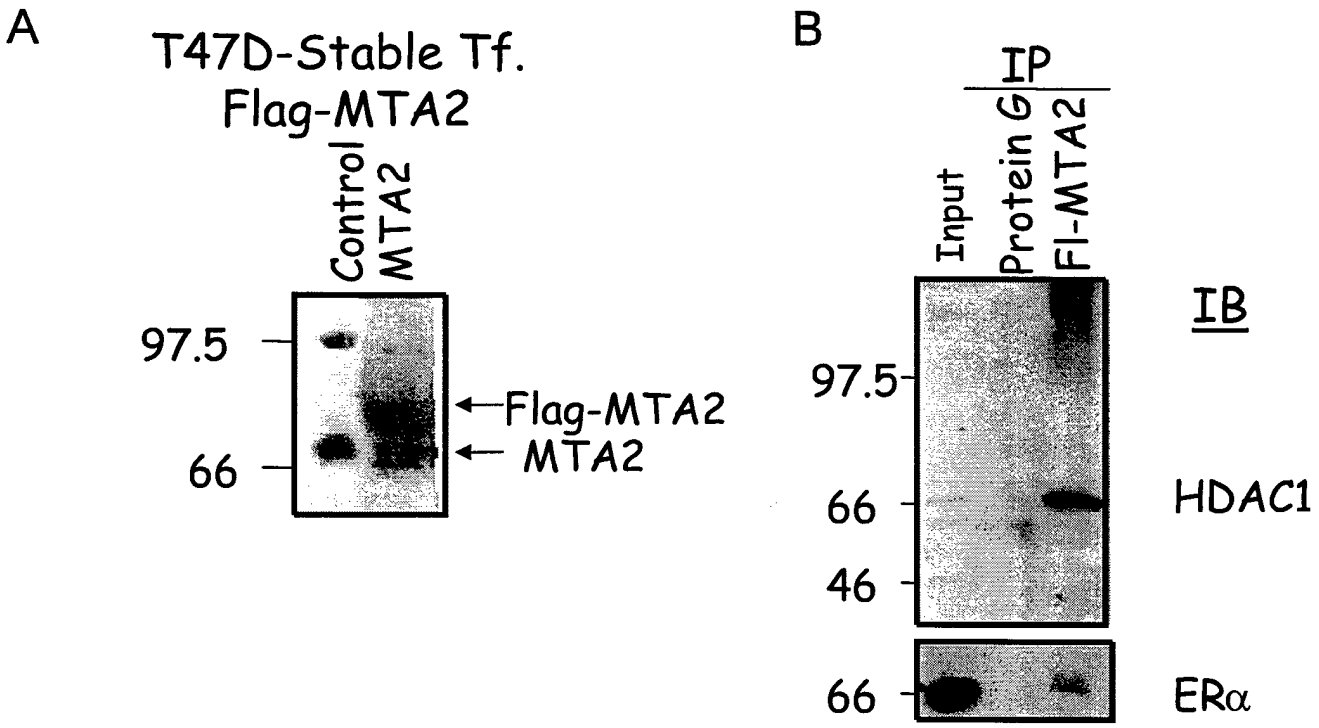
Fig.8. Same as Fig. 4. The different expression vectors and cell lines were indicated in the figures.

Fig.9. Two thousand cells of each of two vector controls (V1, V2) or two MTA1-L1 overexpressing T47D cells were seeded per well of 96 well plates in 100μl phenol red- freeMEM plus 5% charcoal stripped FBS. 24 hours later, increasing amounts of estrogen were added into cells and replaced every two days. The cell proliferation were determined with MTT reagents 7

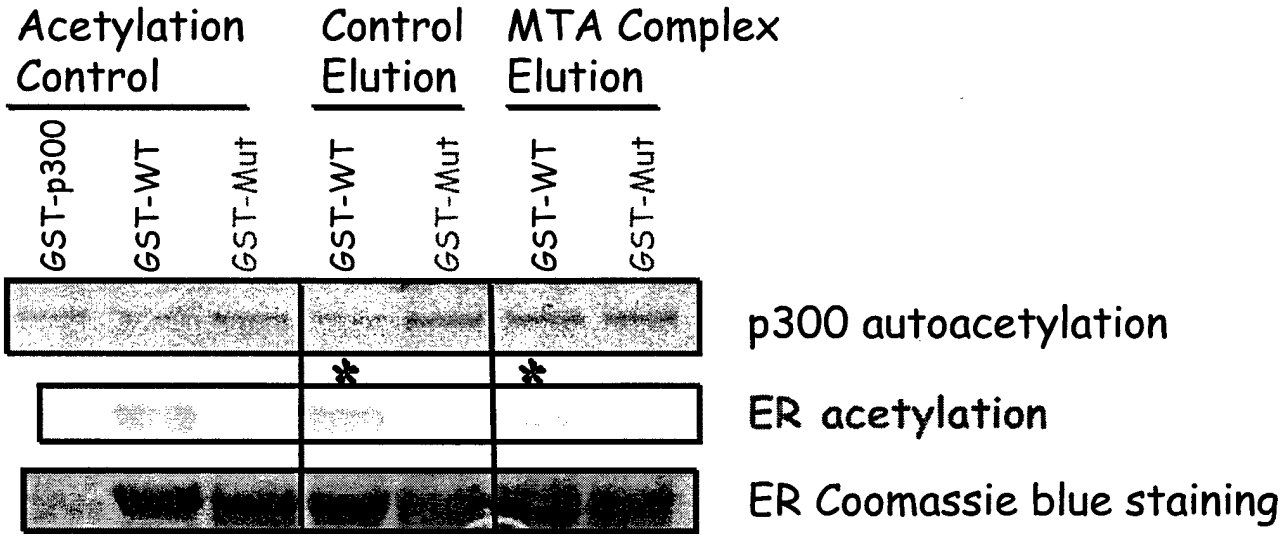
days after the first addition of estrogen. the data showed is an representative of at least three independent experiments. Each treatment were set as 6-repetitions.

Fig.10. Soft agar assay were performed using 6 well plates, 2 ml 0.7% soft agr was plated onto each well as bottom layer, five thousands were suspended in 4 ml 0.6% gel and plated on top of the bottom layer. Cells were than treated with phenol red-free media supplemented with 5% charcoal-stripped plus vehicle, 1nM estrogen, 100 nM 4-hydroxy Tamoxifen, or 1 nM E2 plus 100 nM 4-hydroxy Tamoxifen for 3 weeks. The fresh media were replaced every two days. Then the colony (>50 cells) numbers were counted under microscope. The data shown is a representative of two independent experiments with each treatment set as duplicate.

Figure 1

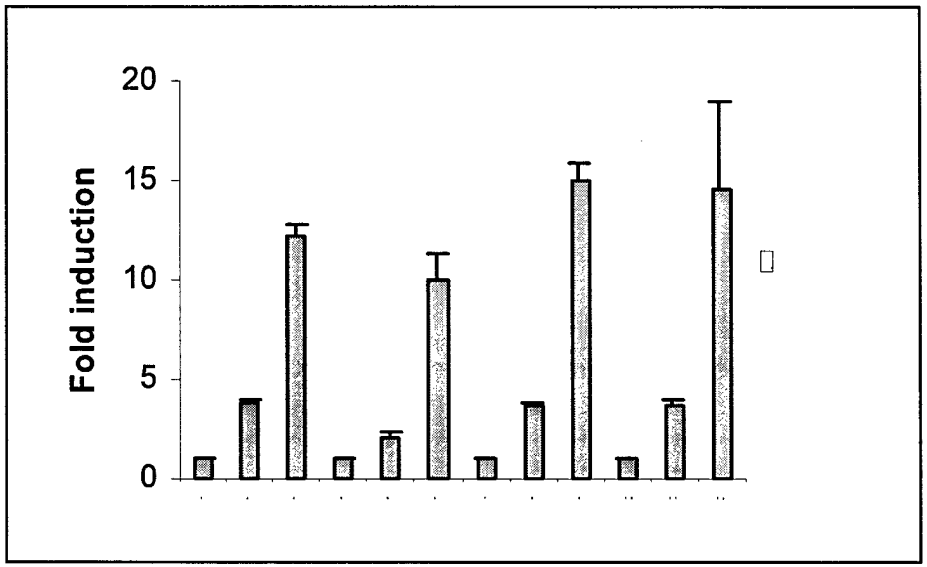

A
T47D-Stable Tf.
Flag-MTA2
Control
MTA2
97.5
66
Flag-MTA2
MTA2
B
IP
Input
Protein G
Fl-MTA2
IB
97.5
66
46
HDAC1
66
ERα

C



MTA2 overexpression leads to deacetylation of ER

Figure 2



Wt-ER	+	+	+	+	+	+	-	-	-	-	-	-
K303R	-	-	-	-	-	-	+	+	+	+	+	+
MTA2	-	-	-	+	+	+	-	-	-	+	+	+
E2	-	+	+	-	+	+	-	+	+	-	+	+
NaB	-	-	+	-	-	+	-	-	+	-	-	+

Figure 3

A

B

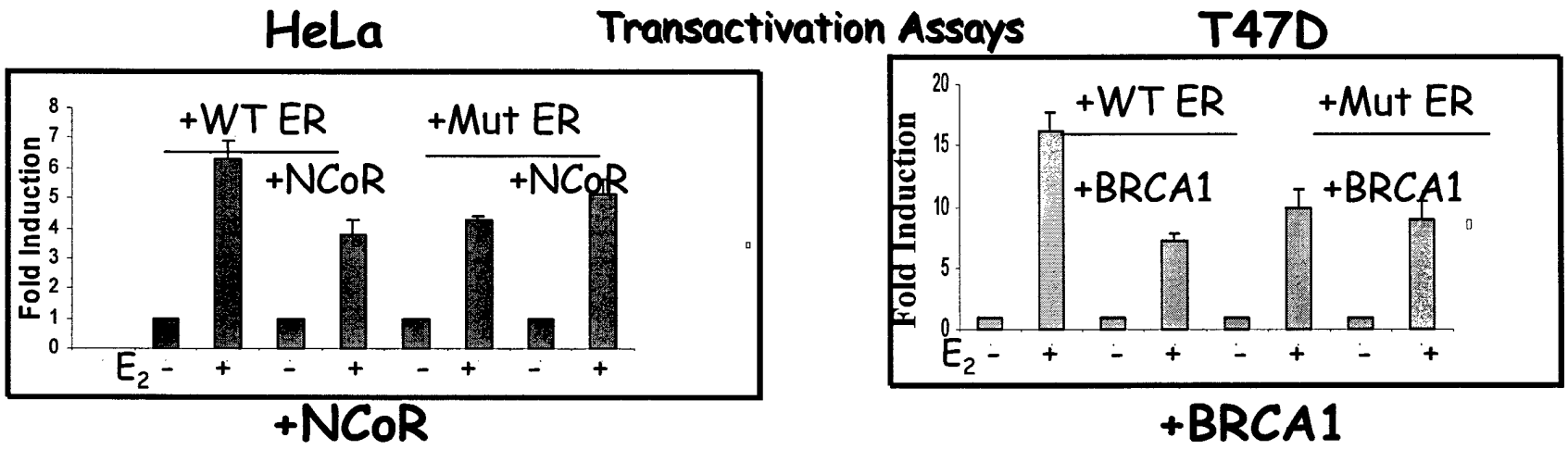
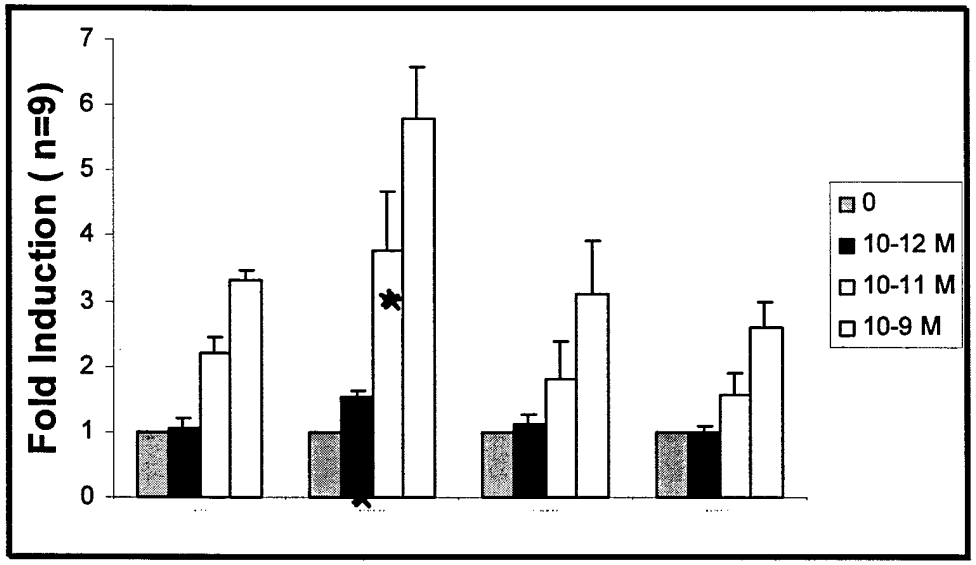

HeLa
Transactivation Assays
T47D
Fold Induction
+WT ER
+Mut ER
+NCoR
+NCoR
+BRCA1
+BRCA1
E2
+NCoR
+BRCA1

Figure 4

A


Fold Induction (n=9)
7
6
5
4
3
2
1
0
0
10-12 M
10-11 M
10-9 M
ERα
WT
K303R
K303M
K303T
E2

B

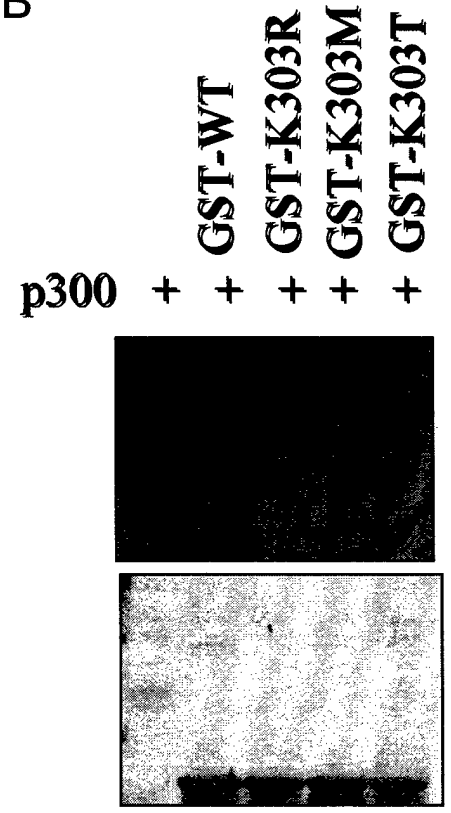
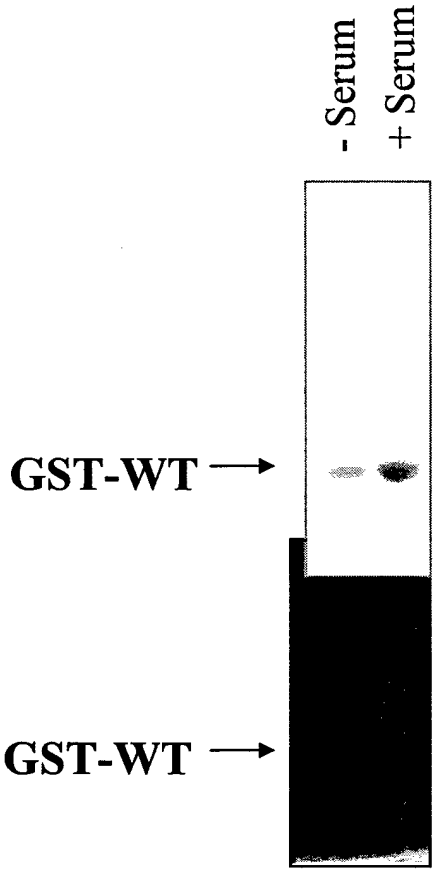

GST-WT
GST-K303R
GST-K303M
GST-K303T
p300 + + + + +

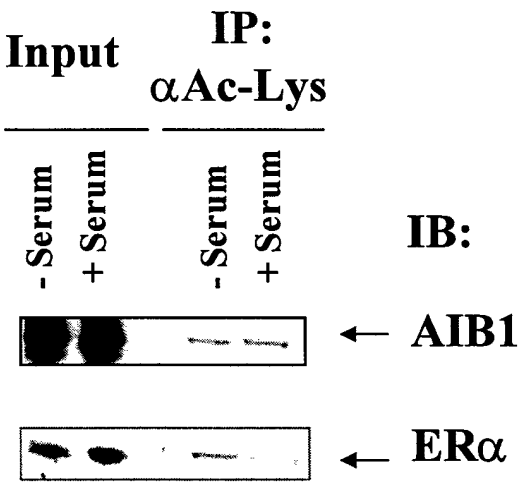
Figure 5

A

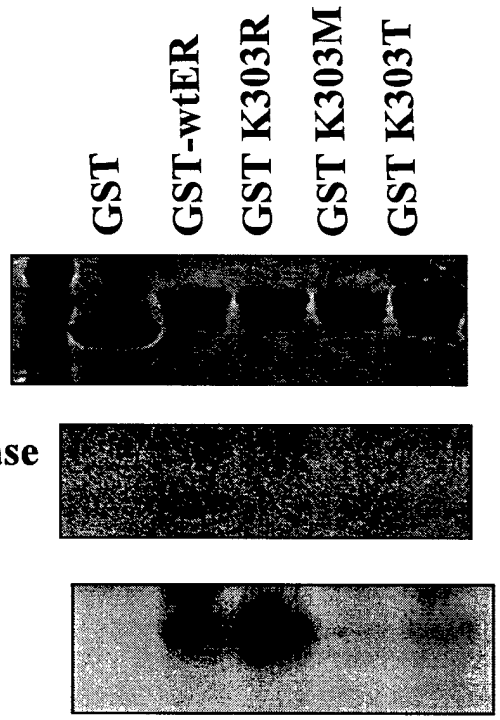


Total Kinases Assay

B



C


GST
GST-wtER
GST K303R
GST K303M
GST K303T
Input
ER-Hinge Autokinase Assay
ER Hinge Total Kinase Activity in MCF-7cells

D

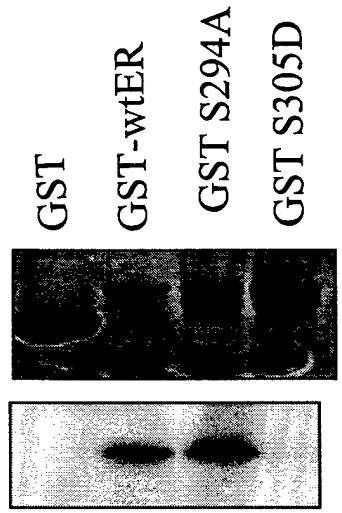
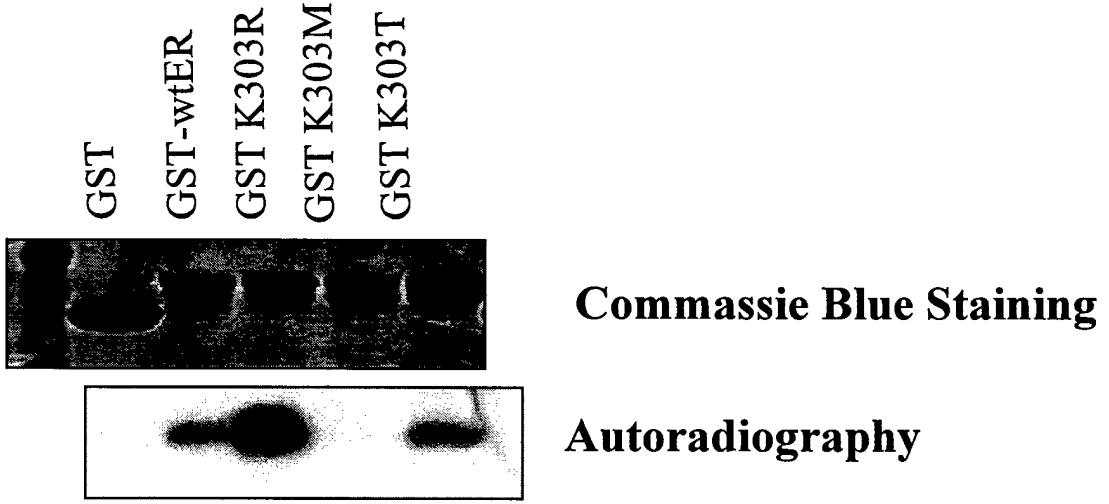

GST
GST-wtER
GST S294A
GST S305D
Input
Total Kinase Assay

Figure 6

A


GST
GST-wtER
GST K303R
GST K303M
GST K303T
Input
Commassie Blue Staining
PKA Assay
Autoradiography

B

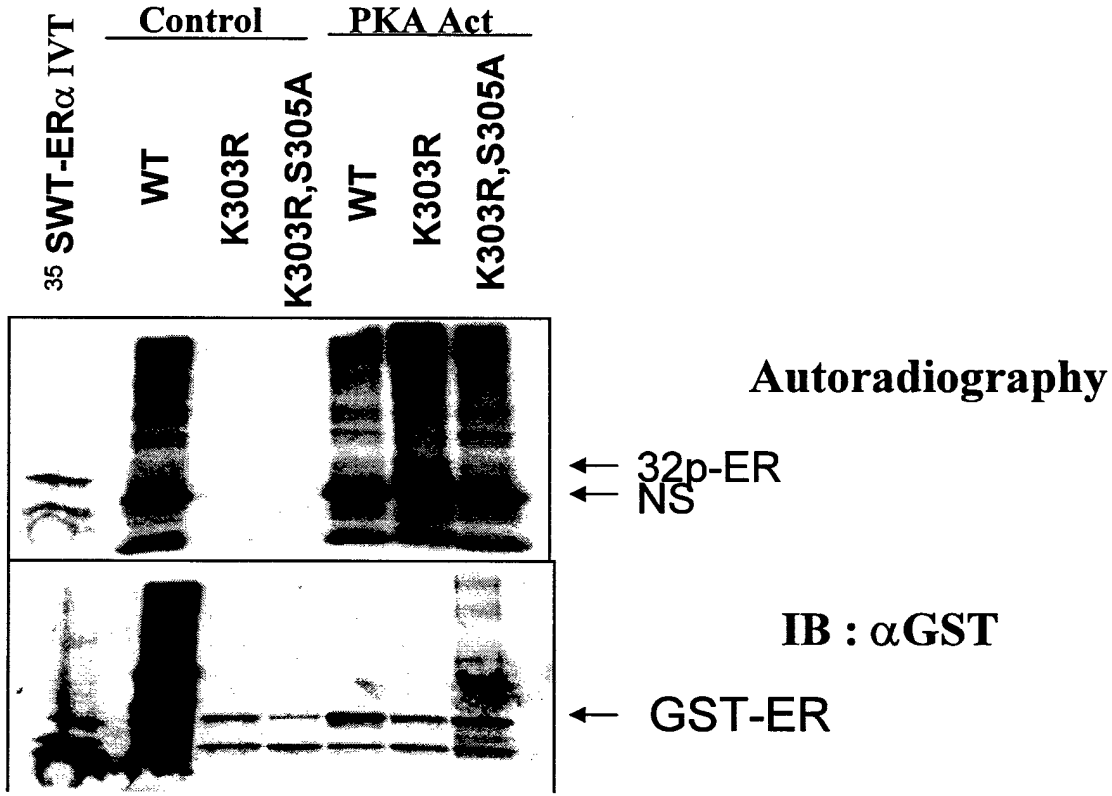
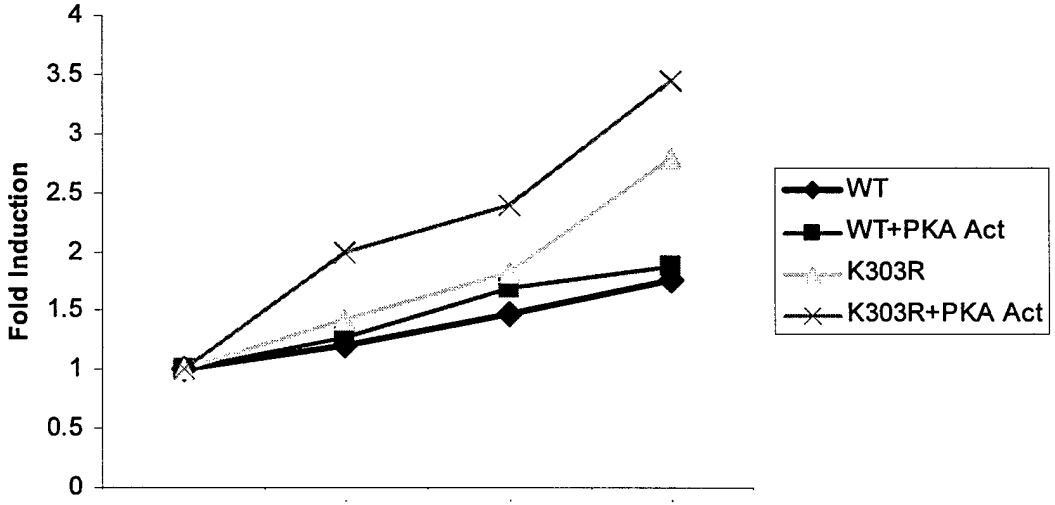

35 SWT-ERα IVT
Control
PKA Act
WT
K303R
K303R,S305A
WT
K303R
K303R,S305A
Autoradiography
32p-ER
NS
IB : αGST
GST-ER

Fig. 7

A.


Fold Induction
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
WT+PKA Act
K303R
K303R+PKA Act

B.

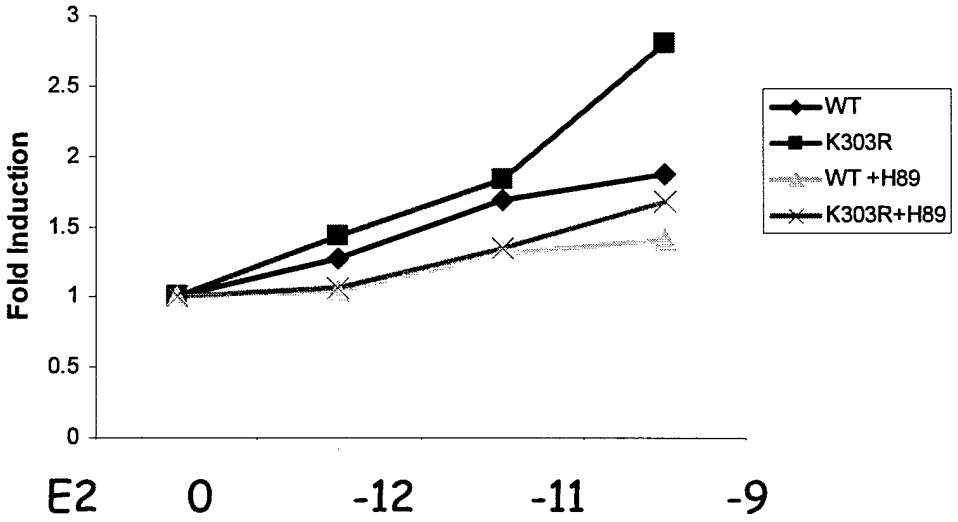

Fold Induction
3
2.5
2
1.5
1
0.5
0
WT
K303R
WT +H89
K303R+H89
E2
0
-12
-11
-9

Figure 8

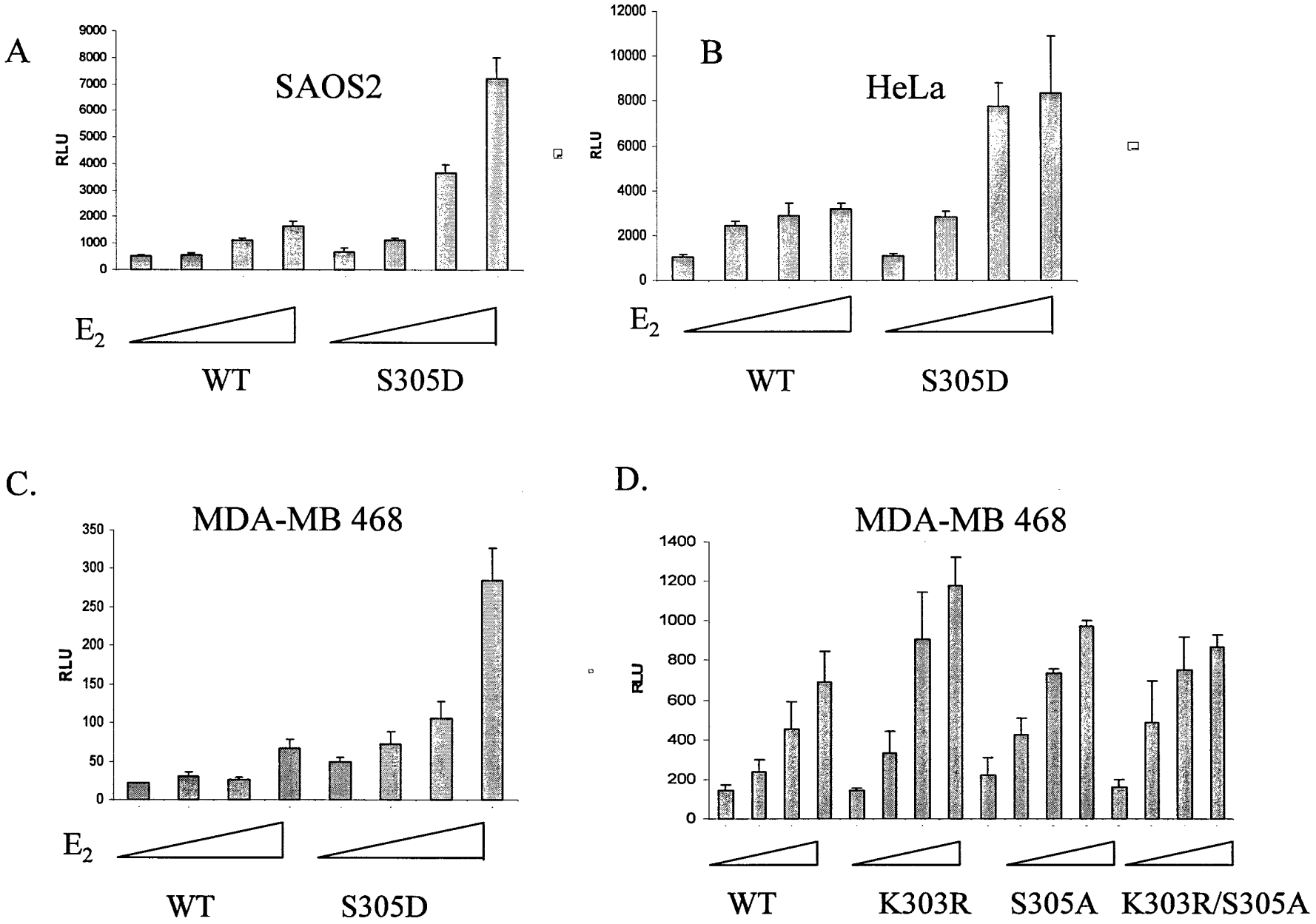


Fig. 5

Figure 9

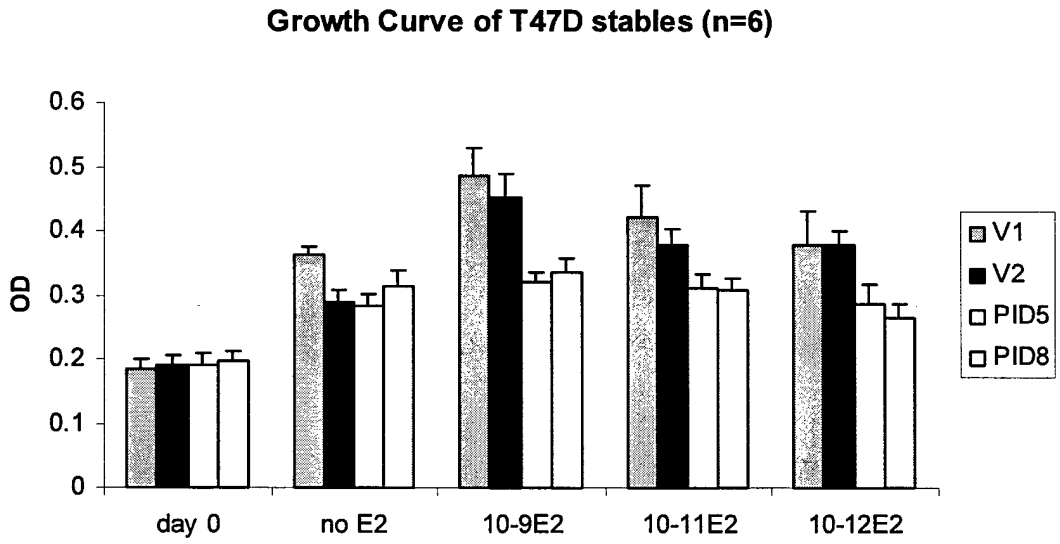

Growth Curve of T47D stables (n=6)
OD
0.6
0.5
0.4
0.3
0.2
0.1
0
day 0
no E2
10-9E2
10-11E2
10-12E2
V1
V2
PID5
PID8

Figure 10